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Atypical Microglial Response to Biodiesel Exhaust in Healthy and Hypertensive Rats

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Abstract

Accumulating evidence suggests a deleterious role for urban air pollution in central nervous system (CNS) diseases and neurodevelopmental disorders. Microglia, the resident innate immune cells and sentinels in the brain, are a common source of neuroinflammation and are implicated in how air pollution may exert CNS effects. While renewable energy, such as soy-based biofuel, is of increasing public interest, there is little information on how soy biofuel may affect the brain. To address this, male spontaneously hypertensive rats (SHR) and normotensive Wistar Kyoto (WKY) rats were exposed to 100% Soy Biodiesel Exhaust (100SBDE; 0, 50, 150 and 500 μg/m^3) by inhalation for 4 h/day for 4 weeks (5 days/week). IBA-1 staining of microglia in the substantia nigra revealed significant changes in morphology with 100SBDE exposure in rats from both genotypes, where the SHR were less sensitive. Further analysis failed to show consistent changes in pro-inflammatory cytokine expression, nitrated protein, and arginase1 expression in brain tissue from either rat strain exposed to 100SBDE. CX3CR1 and fractalkine mRNA expression were

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lower in the striatum of all 100SBDE exposed rats, but greater SBDE exposure was required for loss of fractalkine expression in the SHR. Together, these data support that month-long 100SBDE exposure impacts the basal ganglia with changes in microglia morphology, an impaired fractalkine axis, and an atypical activation response without traditional markers of M1 or M2 activation, where the SHR may be less sensitive to these effects.

Keywords

Biodiesel; Air Pollution; Brain; Microglia; Atypical Activation

1. INTRODUCTION

Renewable energy, such as biofuel, has received increasing public attention. However, current understanding about the health effects of biofuel is limited (Larcombe et al. , 2015), particularly regarding how it might impact the brain. Derived from plant or animal oil through the transesterification of triglycerides with alcohol, biodiesel may be used in place of traditional petroleum diesel fuel sources (Larcombe, Kicic, 2015, Swanson et al. , 2007). Importantly, depending on the source and composition, the exhaust emissions of biofuel pollutants (particulate matter, adsorbed chemicals, and gases) can be lower than traditional mineral fuel diesel exhaust (Mutlu et al. , 2015, Prokopowicz et al. , 2015, Shojaeefard et al. , 2013). While this suggests the potential for health benefits, the effects of each exhaust might be unique to the organ system examined. For example, biodiesel has been shown to result in acute pulmonary inflammation similar to petroleum diesel (Mehus et al. , 2015), but also elevated acute vascular effects (Bass et al. , 2015), significant increased liver and lung tissue damage (Shvedova et al. , 2013), elevated pulmonary and liver oxidative stress (Shvedova, Yanamala, 2013), and more pronounced pro-inflammatory pulmonary responses (Shvedova, Yanamala, 2013). At present, the impact of biofuel in the brain is largely unknown.

Urban air pollution is a complex chemical mixture derived from multiple sources, including engine emissions, coal combustion, biomass burning, and secondary photochemical products. Diesel exhaust is a major constituent of near-road and urban air pollution and is commonly used as a surrogate model of air pollution in health effects studies (Hesterberg et al. , 2010, Ma and Ma, 2002). In the U.S. alone, millions of people are exposed to air pollution levels above safety standards (Mauderly et al. , 2010). Importantly, evidence points to a link between high levels of urban air pollution and several CNS conditions including, elevated stroke incidence (Bedada et al. , 2012, Wellenius et al. , 2012b), increased Autism risk (Becerra et al. , 2013, Roberts et al. , 2013, Volk et al. , 2011, Volk et al. , 2013), decreased cognitive function in the elderly (Power et al. , 2011, Power et al. , 2013, Wellenius et al. , 2012a), elevated Alzheimer’s disease (AD)-risk (Jung et al. , 2015), increased Parkinson’s disease (PD) risk (Kirrane et al. , 2015), and accelerated neurodegenerative disease progression (Kioumourtzoglou et al. , 2015). This is consistent with case studies demonstrating an association of living in highly polluted areas with elevated AD-like neuropathology (Calderon-Garciduenas et al. , 2012, Calderon-Garciduenas et al. , 2004, Calderon-Garciduenas et al. , 2008) and Parkinson’s-disease like

neuropathology in humans (Calderon-Garciduenas et al. , 2011, Calderon-Garciduenas et al. , 2013). As such, it is of pressing public health interest to understand the impact of different sources of air pollution on the brain and their effect on unique genetic backgrounds that might confer vulnerability.

Microglia are the resident innate immune cells in the brain, mandatory for healthy CNS function, and constantly active, both as sentinels surveying the CNS environment (Hickman et al. , 2013) and as “electricians” regulating synaptic communication (Sun et al. , 2014). As sentinels, microglia detect and respond to cellular damage, pathogens, and environmental exposures (Block et al. , 2007), resulting in a shift in phenotype commonly referred to as “activation”. The deleterious response associated with neuron damage and CNS disease is predicted to occur when the microglial pro-inflammatory response is excessive, unregulated, and fails to resolve (Block, Zecca, 2007, Heneka et al. , 2014). Microglia have long been implicated in the neuronal damage occurring in many CNS diseases and conditions, including those impacted by air pollution such as AD (Heppner et al. , 2015), PD (Block, Zecca, 2007), and Autism (Takano, 2015). Thus, microglia and neuroinflammation have been implicated as a common underlying mechanisms through which air pollution may affect diverse CNS conditions (Block and Calderon-Garciduenas, 2009).

Increasing evidence indicates that ambient air pollutants are a prevalent environmental source of chronic neuroinflammation and microglial activation (Costa et al. , 2015). Urban particulate matter (Allen et al. , 2014, Campbell et al. , 2009, Campbell et al. , 2005, Morgan et al. , 2011), O_3 (Santiago-Lopez et al. , 2010), diesel exhaust (Bolton et al. , 2012, Gerlofs-Nijland et al. , 2010, Levesque et al. , 2011a, Levesque et al. , 2011b), and manganese (Antonini et al. , 2009, Elder et al. , 2006) are examples of inhaled pollutants that elevate CNS cytokines and oxidative stress in animal models. Human reports confirm microglia activation and neuroinflammation in individuals living in highly polluted cities (Calderon-Garciduenas, Solt, 2008). Additionally, *in vitro* studies indicate that the individual components of air pollution, especially particulate matter, may directly activate microglia (Block et al. , 2004, Campbell et al. , 2014, Levesque, Taetzsch, 2011b, Morgan, Davis, 2011). Thus, while the specific mechanisms underlying how microglia detect and respond to air pollution remain unresolved, there appears to be a prototypical pro-inflammatory microglial activation phenotype and neuroinflammation linked to several forms of air pollution, particularly diesel exhaust.

In an effort to begin to understand how pollutants from biodiesel exhaust may affect the brain, we addressed the effects of month-long exposure to soy biodiesel exhaust on the neuroinflammation phenotype in the brains of normal and hypertensive rats, including morphology, M1 markers, and M2 markers. The spontaneously hypertensive rats have been previously reported to be more vulnerable to the extra-pulmonary effects of air pollution (Saxena et al. , 2009) and were originally predicted to be more sensitive to the CNS effects of biodiesel exposure.

2. METHODS

2.1. Reagents

The polyclonal antibody against the Ionized Calcium-binding adapter molecule-1 (IBA-1) microglial marker was purchased from Wako (Richmond, VA). The Biotinylated goat anti-rabbit secondary antibody was purchased from Vector Laboratories (Burlingame, CA). ELISA kits were purchased from R&D Systems (Minneapolis, MN). All other reagents were procured from Sigma-Aldrich (St. Louis, MO).

2.2. Animals

Twelve, fourteen week old male Spontaneously Hypertensive rats (SHR) and normotensive Wistar Kyoto (WKY) rats were purchased from Charles River Laboratories (Raleigh, NC). SHR are a model of cardiovascular disease frequently employed to discern the underlying mechanisms of the cardiopulmonary health effects of PM, where SHR are more vulnerable to these extra-pulmonary effects of air pollution (Saxena, Gilmour, 2009). Animals were acclimated to the housing facility for 1 week before studies began. Rats were housed in an AAALAC-accredited housing facility and maintained at 20–24°C and on a 12h light: dark schedule. All animal experiments were approved by the US EPA IACUC and the National Institutes of Health guidelines were followed. All animals were treated humanely and with regard for alleviation of suffering.

2.3. Biodiesel Exhaust Exposure

A 100% soy biodiesel fuel (100SBDE) was purchased from Piedmont Biofuels (Pittsboro, NC). This fuel was used to power a Yanmar L70 diesel engine (Adairville, GA) and Pramac E3750 generator (Marietta, GA). This single cylinder, 320-cm^3 engine ran at 5.8 hp (4.3 kW) with a continuous load, 3600 rpm. The engine exhaust (~ 85 L/min) was diluted with HEPA and charcoal filtered room air (~7:1 dilution) and was directed to three 984 L Hazelton model 1000 inhalation exposure chambers located in an adjacent inhalation exposure laboratory. Additional HEPA filtered room air was introduced for secondary dilution to the desired exposure concentrations. The aerosol concentration in each chamber was continuously monitored using tapered element oscillating microbalances (TEOM, Rupprecht and Patashnick Co., series 1400, Albany, NY). Particle-size distributions were measured using a scanning mobility particle sizer (SMPS, TSI Inc., model 3080, St. Paul, MN) for all chambers. Target exposure concentrations of 50, 150, and 500 $\mu g/m^3$ were achieved concurrently in the three chambers by manually adjusting the secondary dilution according to the TEOM determined mass concentration. The fourth Hazelton chamber was used as a control, receiving only HEPA/charcoal filtered room air. Inhalation chamber temperatures, pressures, and relative humidity were maintained hourly during exposure. The flow rate of 283 L/min (10 ft^3/min) for all chambers was maintained, which is sufficient for approximately 17 air changes per hour. Also, all chambers were continuously monitored for oxygen (O_2, California Analytical Instruments, model 200, Orange, CA,), carbon monoxide (CO, Thermo Electron Instruments, model 48, Fitchburg, WI,), sulfur dioxide (SO_2, Thermo Environmental Instruments, model 43C, Franklin, MA,), nitric oxide and total nitrogen oxides (NO and NO_x, Thermo Scientific, model 42i-HL, West Palm Beach, FL). Nitrogen

dioxide (NO_2) levels were obtained by subtracting NO values from total NO_x. Further details are provided in previous publications (Mutlu et al.,2015; Bass et al., 2015).

In brief, Rats were exposed for 4 h/day for 4 weeks (5 days/week) to 100% Soy Biodiesel Exhaust (100SBDE) with particulate matter concentrations of 0, 50, 150 and 500 μg/m^3, which are levels higher than typically encountered in ambient air, but may be achieved during heavy traffic or occupational situations. The exhaust characterization for 100SBDE was previously reported in detail and compared to values from exhaust obtained from petroleum diesel fuel (Mutlu, Nash, 2015), where 100SBDE PM concentrations were 30% lower than traditional petroleum diesel fuel exhaust. When emissions were diluted with air to control equivalent PM mass concentrations, 100SBDE exhibited lower CO and slightly higher NO concentrations when compared to the traditional petroleum diesel fuel exhaust (Mutlu, Nash, 2015). Further, the organic/elemental carbon ratios and oxygenated methyl esters and organic acids were higher for 100SBDE and it exhibited unimodal-accumulation mode particle-size distributions, with mildly higher concentrations of slightly larger particles when compared to exhaust composition from traditional petroleum diesel fuel (Mutlu, Nash, 2015). Only rats exposed to filtered air and 100SBDE were used in this study, where one day following the last day of exposure, rats were anesthetized with an overdose of sodium pentobarbital (Virbac AH, Inc., Fort Worth, TX; 50–100 mg/kg, ip), and the left hemisphere of the brain was immediately dissected (striatum and remaining brain) for protein and mRNA analyses and the right hemisphere was fixed for histology. The striatum was targeted due to our previous reports demonstrating the midbrain (Levesque, Surace, 2011a, Levesque, Taetzsch, 2011b), and potentially the basal ganglia, may be more sensitive to the pro-inflammatory effects of diesel exhaust.

2.4. Quantitative Reverse Transcriptase Polymerase Chain Reaction

Total RNA was extracted from the rat brain striatum using Trizol (Invitrogen Life Technologies, Grand Island, NY) according to manufacturer's instructions. The RNA was treated with Ambion DNase I (Invitrogen Life Technologies, Grand Island, NY), which was subsequently removed with Qiagen RNeasy RNA cleanup kit (Qiagen, Germantown, MD). Reverse transcription of RNA (0.3 – 1.0 μg/sample) was performed with iScript Reverse Transcription Supermix (BioRad, Hercules, CA) according to manufacturer's instructions. SsoFast Evagreen Supermix (BioRad, Hercules, CA) and 500 nM forward and reverse primers were used to carry out quantitative PCR on a CFX96 (BioRad, Hercules, CA) real-time PCR detection system, per manufacturer's instructions. Cycling parameters were 1 cycle at 95ºC for 5 min, 40 cycles of 95ºC (5 sec.) and 56ºC (5 sec.) followed by a melt curve measurement consisting of 5 second 0.5ºC incremental increases from 65ºC to 95ºC. The primer sequences are listed in Table S1.

2.5. Protein Isolation: Brain Homogenate

Remaining brain tissue was homogenized as general brain homogenate. Protein was isolated by suspending frozen tissue in 10 volumes of lysis buffer (Cytobuster Protein Extraction Reagent; EMD Chemicals; Darmstadt, Germany) with 10 μL/mL HALT protease inhibitor and 10 μL/mL EDTA. Samples were homogenized using a motorized pellet mixer and then

centrifuged for 5 minutes at 5000 g. The protein concentration of the resulting supernatant was determined using a BCA protein assay (ThermoScientific; Rockford, IL).

2.6. Immunohistochemistry

The right hemisphere of the brain was fixed in 4% paraformaldehyde for 2 days and cryoprotected in 30% sucrose. Coronal sections (40 μm) of the midbrain region containing the substantia nigra pars compacta (SNpc) were collected with a freezing stage microtome (Microm HM 450, Thermo Scientific, Waltham, MA). Dopaminergic neurons were stained to confirm that sectioned samples across rat brain were in the same frame of the SNpc, and were recognized with an anti-tyrosine hydroxylase (TH, 1:1000) rabbit polyclonal antibody (Millipore Billerica, MA) and microglia were stained with a polyclonal rabbit anti-ionized calcium-binding adaptor molecule-1 (IBA-1, 1:1000) antibody (Wako, Richmond, VA). Free-floating brain slices were treated with 1% H_2O_2, washed twice for 10 min with phosphate buffered saline (PBS), incubated 20 min with a blocking solution (PBS containing 1% bovine serum albumin, 4% goat serum, 0.4% Triton X-100) and incubated overnight at 4°C with primary antibody diluted 1:1000 in DAKO antibody diluent. Slices were then washed twice in PBS, incubated with biotinylated anti-rabbit antibody (Vector Laboratories, Burlingame, CA) for 1 h, washed twice in PBS, and incubated with Vectastain ABC Kit (Vector Laboratories, Burlingame, CA) reagents according to manufacturer's instructions. Staining was visualized using 3,3'-diaminobenzidine and urea-hydrogen peroxide tablets (Sigma-Aldrich, St. Louis, MO). Images were captured with an Olympus BX51 microscope (Olympus America, Center Valley, PA).

2.7. Immunoblotting

Ten micrograms of protein from each sample was electrophoresed on a 12% SDS-PAGE gel. Samples were transferred to nitrocellulose membranes by semi-dry transfer, blocked with 5% nonfat milk for 1 hr. at 24°C, followed by incubation overnight with the anti-GAPDH (1:1000), anti-P38 (1:1000) and anti-phosphorylated P38 (1:1000) antibodies at 4°C (Cell Signaling Technologies, Danvers, MA), where all antibodies have been reported to cross-react with the rat proteins. Blots were then incubated with horseradish peroxidase-linked mouse anti-rabbit (1:5000) or goat anti-mouse (1:5000) for 1 h (24°C) and ECL+Plus reagents (Amersham Biosciences Inc., Piscataway, NJ) were used as a detection system. Band density was quantitated with ImageJ (Abramoff et al. , 2004) and analyzed as a ratio of total P38 and phosphorylated P38 bands. Results are reported as a percent change from control.

2.8. Cytokine ELISAs

Fractalkine and TNFα protein concentrations in 100μg/well of brain homogenate protein was measured using a commercial enzyme-linked immunosorbent assay (ELISA) kit from R&D Systems (Minneapolis, MN).

2.9. Nitrotyrosine ELISA

The amount of nitrated proteins was measured using 100 μg/well of brain homogenate with a commercial enzyme-linked immunosorbent assay (ELISA) kit from Millipore (Temecula, CA), per the manufacturer protocol.

2.10. Statistical Analysis

Data are expressed as representative images, raw values, fold change, and the percentage of control, where control values were set to either 100% or 1 accordingly. The treatment groups are expressed as the mean and SEM, where statistical significance was assessed with a one-way or two-way ANOVA, followed by Bonferroni's post hoc analysis (GraphPad Prism v 6.0). The accepted level of statistical significance was $P < 0.05$.

3. RESULTS

3.1. Microglia in the Substantia Nigra Change Morphology in Response to Biodiesel Exposure

Previous reports indicate that microglia in the substantia nigra are activated and change morphology in response to month-long inhalation to petroleum-based diesel exhaust (Levesque, Taetzsch, 2011b). Using a similar histopathological approach, here we revealed that microglia also demonstrate activated morphology in the substantia nigra in response to 100SBDE (Figure 1). Interestingly, the representative images in Figure 1 suggest that higher concentrations of 100SBDE may be required for changes in morphology to occur in the SHR compared to WKY rats. Activated morphology in response to 100SBDE is denoted by larger cell bodies, fewer processes, and thicker remaining processes (Figure 1). Thus, microglia in the substantia nigra are clearly able to detect and respond to month-long biodiesel exhaust exposure.

3.2. Biodiesel Exposure Fails to Consistently Impact Markers of Traditional M1/M2 Activation in the Brain

Microglial activation and healthy neuroinflammation typically exists on a spectrum of pro-inflammatory (M1) or alternative (M2) responses (Boche et al. , 2013, Durafourt et al. , 2012, Jang et al. , 2013). What is frequently characterized as M1 activation is associated with the upregulation of pro-inflammatory mediators (ex. TNFα and IL-6), the production of reactive oxygen species (ROS, ex. H_2O_2 and $ONOO^-$) (Block, Zecca, 2007), and initiating signaling pathways associated with neuroinflammation (ex. p38 MAP Kinase phosphorylation) (Bachstetter and Van Eldik, 2010). In healthy activation responses, the initial M1 response is typically followed by a secondary M2 activation that is important for wound healing and resolving inflammation, which is marked by the expression of factors such as Arginase1 (ARG-1) (Boche, Perry, 2013, Durafourt, Moore, 2012, Mills, 2012). In an effort to discern how microglia were responding to the 100SBDE exposure and to characterize the brain pro-inflammatory milieu, several M1/M2 markers were assessed. In contrast to our previous reports of generalized whole brain neuroinflammation with petroleum-based diesel exhaust exposure (Levesque, Taetzsch, 2011b), 100SBDE exposure resulted in no significant changes in p38 MAP Kinase phosphorylation (Table S3, $P>0.05$)

or TNFα protein (Table S4, P>0.05) in brain homogenates, regardless of genotype. Exposure to 100SBDE did elevate brain nitrotyrosine levels only in WKY rats, but this was not a dose-dependent response (Table S2 P<0.05). Analysis of mRNA from the striatum revealed no consistent dose response for TNFα (Table S5, P>0.05), IL-6 (Table S6, P>0.05), or ARG-1 (Table S7, P>0.05) gene expression due to 100SBDE exposure, regardless of genotype. While IBA-1 mRNA showed no significant gene expression changes in the striatum (Table S8, P>0.05), the microglia-specific receptor CX3CR1 showed significant downregulation, regardless of genotype (Figure 2, P<0.05). Interestingly, there was a genotype by environment interaction in striatum fractalkine mRNA levels with 100SBDE exposure, where SHR rats appear to be less sensitive to 100SBDE-induced reduction of fractalkine expression (Figure 3, P<0.05). Thus, the data indicate the microglial activation phenotype associated with the changes in morphology is independent of genotype and is atypical, where neuroinflammation is absent and there is no discernable effect on the traditional M1/M2 markers measured. However, unique to this activation phenotype, it appears that CX3CR1 may decrease in microglia in the striatum without affecting other myeloid specific markers. In addition, fractalkine expression (the ligand for CX3CR1) is decreased in the striatum in both strains, where the WKY rats are the most sensitive to these effects, suggesting a potential impairment of fractalkine signaling with biodiesel exposure.

4. DISCUSSION

As sentinels, microglia detect and respond to diverse stimuli that reach the brain, which includes responding to several sources of urban air pollution. Our previous reports document a pro-inflammatory microglial response linked to generalized neuroinflammation in response to diesel exhaust from traditional petroleum-based fuel (Levesque, Taetzsch, 2011b). Here, we demonstrate that microglia have a unique response to diesel exhaust exposure from a different source. More specifically, our current findings indicate that microglia detect biodiesel exhaust and change morphology in the substantia nigra (Figure 1) without consistent evidence of generalized neuroinflammation, oxidative stress, nor changes in M1 or M2 marker gene expression (Tables S2–8). Importantly, there was also no evidence to suport changes in microglia number, which is supported by a lack of any effect of biodiesel exhaust on striatum IBA-1 gene expression (Table S8). However, CX3CR1 was downregulated in all strains, to further differentiate this atypical activation phenotype (Figure 2).

Together, these findings indicate that the CNS response to 100SBDE breaks from the prototypical pro-inflammatory response we have observed with exposure to urban pollution from several sources. With regards to the microglial activation phenotype, it is important to note that the presence of M1 or M2 markers may reveal little about the beneficial or deleterious nature of microglial function when assessed without knowledge of the appropriate kinetic response (Taetzsch et al. , 2015), and the healthy vs. pathological microglial response to urban air pollution is poorly understood. Notably, fractalkine expression was reduced in the striatum of both the SHR and WKY rats, where it took less biodiesel to cause this effect in WKY rats (Figure 3). As such, this suggests biodiesel may perturb the fractalkine axis in the striatum. At present, it is premature to characterize these CNS responses to biodiesel as toxic or benign, as the exposure was short term and the

analyses were not all inclusive. To begin to address this issue, further inquiry will require assessment of chronic effects, including those occurring across a lifetime, which is more similar to human exposure to urban air pollution.

The reduction of CX3CR1 in the brain (Figure 2) without the modification of other myeloid cell markers, like IBA-1 (Table S8), implicates cellular downregulation of this key receptor in microglia. CX3CR1 is a myeloid specific receptor involved in microglial chemotaxis, general microglial function, and is the receptor for fractalkine, a factor produced by neurons for neuron-microglia communication in health and injury (Limatola and Ransohoff, 2014). The biodiesel-induced CNS reduction of CX3CR1 paired with pronounced changes in microglia morphology (Figure 1) were initially surprising, as deficits in CX3CR1 have been previously associated with early impaired changes in microglia morphology in response to an activator/stressor (Hellwig et al. , 2015). However, the consequences of CX3CR1 reduction for CNS function and health is unclear due to conflicting reports in the literature. For instance reduced CX3CR1 has been associated with disease such as schizophrenia (Bergon et al. , 2015) and impairment of cognitive function (Rogers et al. , 2011) as well as neuroprotection (Merino et al. , 2015), increased beneficial microglial phagocytic activity (Merino, Muneton-Gomez, 2015), and other protective effects from reducing the deleterious microglial response to fractalkine (Merino, Muneton-Gomez, 2015). However, recent findings indicate that downregulation of CX3CR1 may have a more ominous consequence in chronic damage, as has been reported for neurotrauma (Zanier et al. , 2015). As such, ongoing research will be focused on understanding the role of fractalkine and CX3CR1 on the effects of air pollution in the brain.

In summary, these data reveal an atypical activated microglial response to biodiesel exhaust, where hypertensive rats appear to be less sensitive to these effects. At present, it is unclear whether this microglial/CNS response is benign, beneficial, or deleterious. The underlying mechanisms and the consequences of this unusual response remains of significant scientific interest in the ongoing effort to understand the potential impact of urban air pollution on CNS health.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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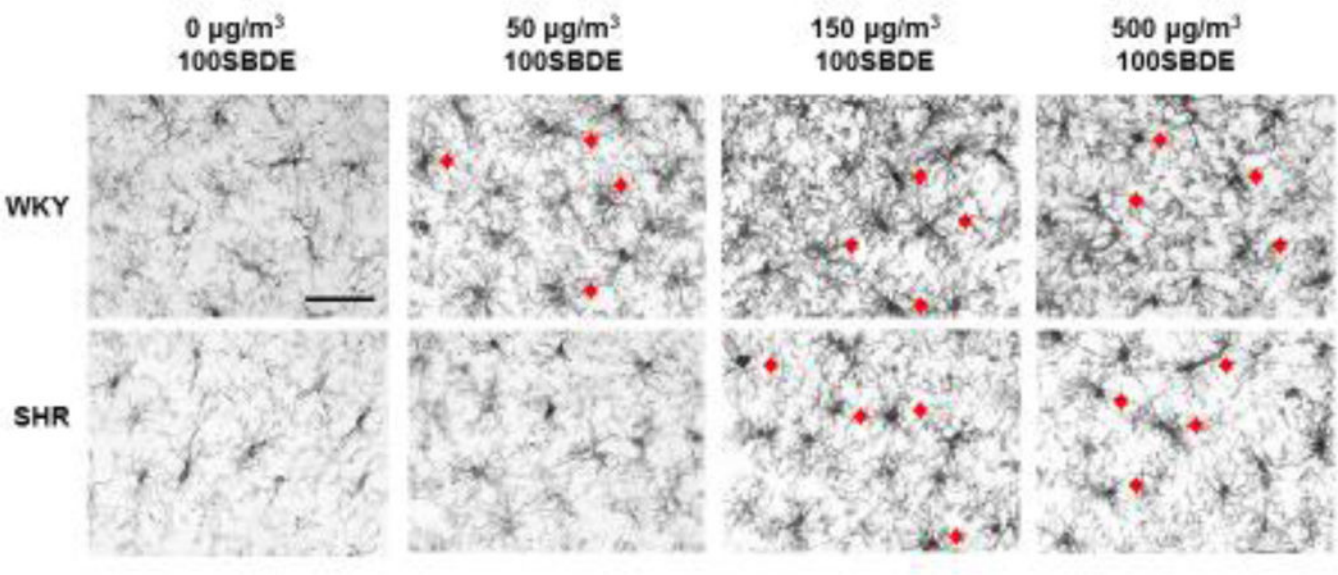

Figure 1. Changes in Microglia Morphology in Response to Soy Biodiesel Exhaust in SHR and WKY Rats.

Young adult male Spontaneously Hypertensive rats (SHR) and normotensive Wistar Kyoto (WKY) rats were exposed for 4 h/day for 4 weeks (5 days/week) to 100% Soy Biodiesel Exhaust (100SBDE; 0, 50, 150 and 500 µg/m^3). Three coronal sections (40 µm) from the substantia nigra per animal were stained with the IBA-1 antibody. Representative images of the substantia nigra were taken at 40X, the scale bar depicts 50 µM, and the red arrows depict activated microglia morphology (N=3).

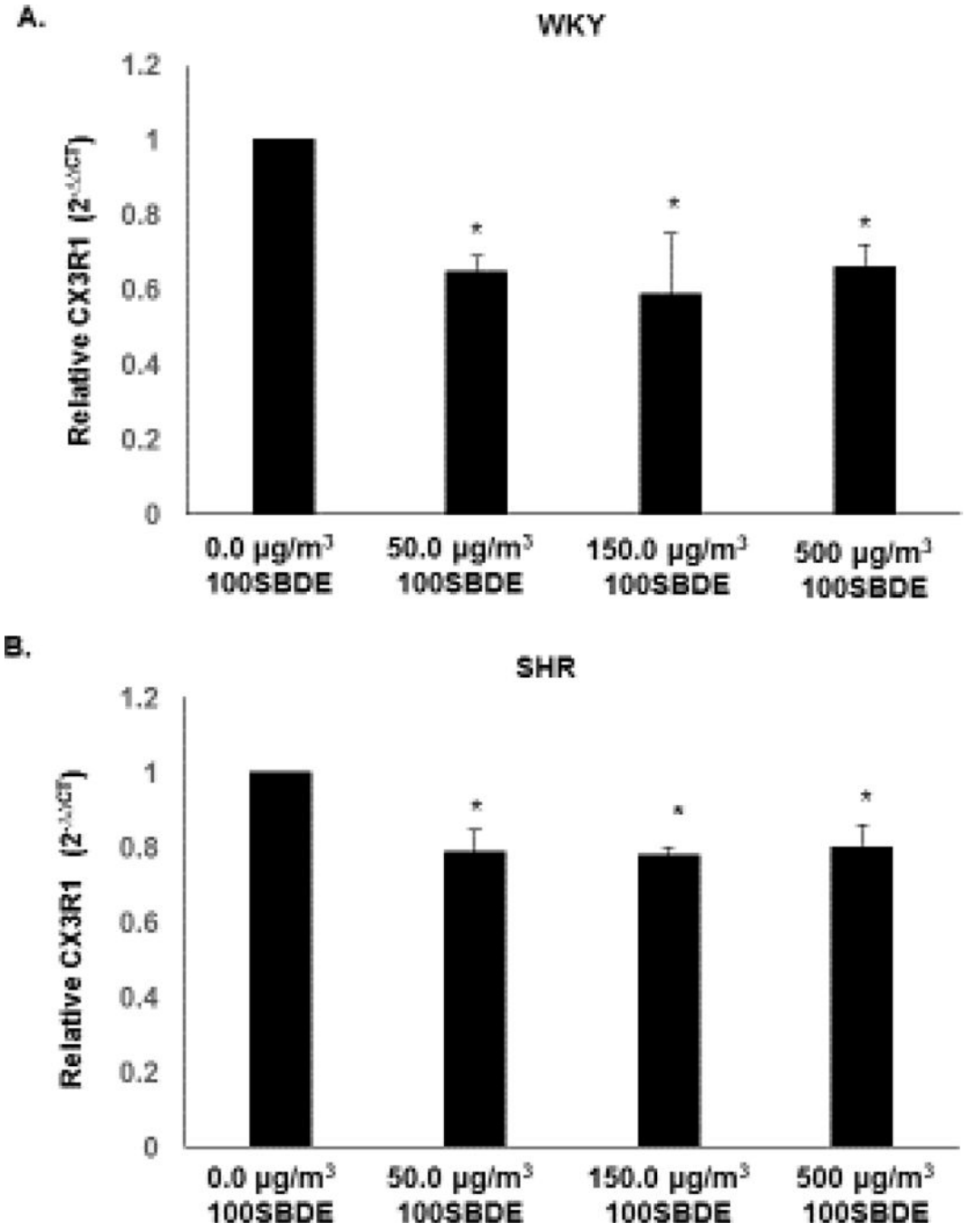

Figure 2. Striatum Changes in Fractalkine in Response to Soy Biodiesel Exhaust in SHR and WKY Rats.

Young adult male Spontaneously Hypertensive rats (SHR) and normotensive Wistar Kyoto (WKY) rats were exposed to 100% Soy Biodiesel Exhaust (100SBDE; 0, 50, 150 and 500 µg/m^3) for 4 h/day for 4 weeks (5 days/week). Striatum fractalkine mRNA expression was evaluated by quantitative RT-PCR, values were normalized to GAPDH using the $2^{-\Delta\Delta CT}$ method. An asterisk indicates significant decrease ($P<0.05$) from 0.0 µg/m^3 control and an "† "indicates a significant difference between the rat strains ($P<0.05$, n=3).

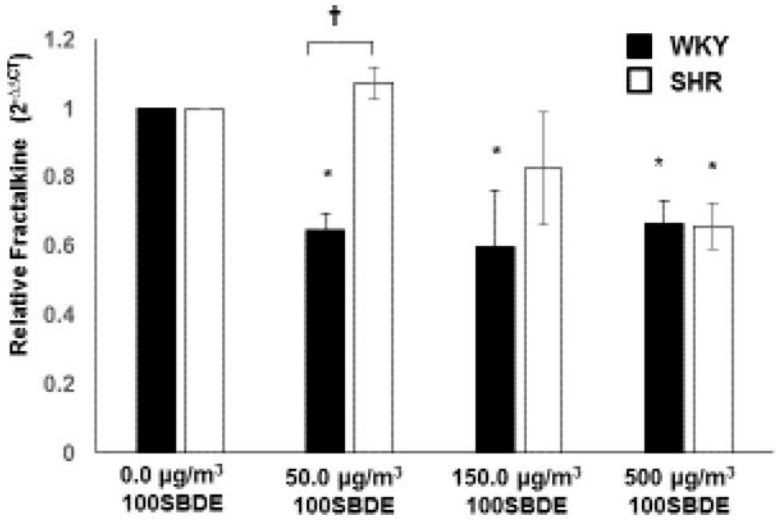

Figure 3. Striatum Changes in Fractalkine Receptor (CX3CR1) in Response to Soy Biodiesel Exhaust in SHR and WYK Rats.

Young adult male Spontaneously Hypertensive rats (SHR) and normotensive Wistar Kyoto (WKY) rats were exposed to 100% Soy Biodiesel Exhaust (100SBDE; 0, 50, 150 and 500 µg/m^3) for 4 h/day for 4 weeks (5 days/week). Striatum CX3R1 mRNA expression was evaluated by quantitative RT-PCR, values were normalized to GAPDH using the $2^{-\Delta\Delta CT}$ method. An asterisk indicates significant decrease ($P<0.05$) from 0.0 µg/m^3 control in both the **A**.WKY rats and **B**. SHR rats.